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Matters Arising

The Human p62 cDNA Encodes Sam68 and Not the RasGAP-Associated p62 Protein

The p62 protein is tyrosine (Tyr) phosphorylated in cells expressing activated Tyr kinases (Ellis et al., 1990) and associates with RasGAP in a phosphotyrosine (pTyr)- and SH2 domain–dependent fashion (Marengere and Pawson, 1992; Moran et al., 1990). The putative p62 protein was purified from v-Src-transformed murine fibroblasts using anti-pTyr antibodies and oligonucleopeptide probes based on peptide sequences obtained to isolate a human cDNA, *hump62* (GenBank accession number M88108; Wong et al., 1992). We and others (Fumagalli et al., 1994; Taylor and Shalloway, 1994; Weng et al., 1994) recently identified Sam68 (Courtneidge and Fumagalli, 1994), a mitotic substrate of Src. Sequence analysis of peptides from murine Sam68 showed that it was highly homologous to Hump62. Yet Sam68 and p62 have distinct properties: p62 is not associated with Src in interphase or mitosis; conversely, Sam68 does not associate with RasGAP and is not Tyr phosphorylated in interphase cells (Fumagalli et al., 1994). Here we show that the *hump62* cDNA encodes Sam68, but not p62, and further that Sam68 and p62 are unlikely to be related proteins.

We used antibodies generated to regions of Hump62 (anti-P, anti-C, anti-KH, and anti-ESK; see Figure 2A) and NIH 3T3 cells transfected with a hemagglutinin (HA) epitope–tagged *hump62* cDNA (3.3 cells) to investigate the relationship between Sam68 and p62. Extracts of quiescent and stimulated 3.3 cells were immunoprecipitated with the different antibodies and analyzed by immunoblotting with anti-pTyr antibodies (Figure 1A, top). RasGAP-associated p62 was detected in quiescent and stimulated cells, but it was not immunoprecipitated by either anti-HA or anti-P antibodies. Conversely, a 68 kDa protein was detected in anti-HA and anti-P but not anti-GAP immunoprecipitates reprobed with anti-HA (Figure 1A, bottom). The anti-HA antibodies also immunoprecipitated a Tyr-phosphorylated protein of approximately 68 kDa from mitotic but not asynchronous extracts (Figure 1B, top). In addition, anti-C antibodies (see Figure 2A) recognized two proteins of approximately 68 kDa that were Tyr phosphorylated only in mitotic cells. (Presumably, the larger protein corresponded to the product of the epitope-tagged *hump62* cDNA, and the smaller protein corresponded to endogenous Sam68.) When the same blot was reprobed with anti-HA antibodies, only a 68 kDa protein was detected in anti-HA and anti-C immunoprecipitates from both asynchronous and mitotic cells (Figure 1B, bottom). Together these results demonstrate that the product of the *hump62* cDNA has a molecular mass of 68 kDa and the properties of Sam68 (see also Taylor et al., 1995).

We reported previously that a commercial antibody against the central portion of the predicted Hump62 protein recognized both Sam68 and a 62 kDa protein, leading us to conclude that Sam68 and p62 were antigenically related (Fumagalli et al., 1994). However, we were unable to detect p62 with other batches of this antibody (data not shown), consistent with reports from others (Ogawa et al., 1994; Taylor and Shalloway, 1994). All four antibodies we generated against different parts of the *hump62* coding sequence (Figure 2A) immunoblotted a 68 kDa protein (Figure 2B): none detected proteins of 62 kDa. Three antisera subsequently tested on metabolically labeled cells also reacted specifically with Sam68 but not p62 (Figure 2C). Finally, although

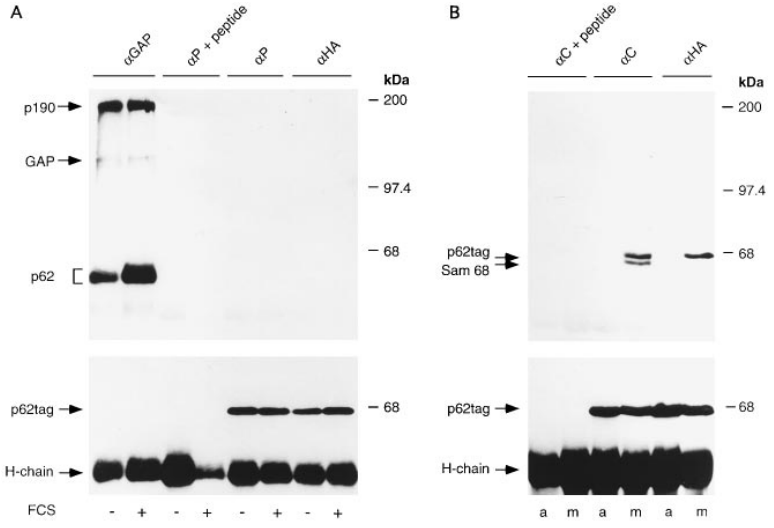

Figure 1. The *hump62* cDNA Encodes Sam68 and Not RasGAP-Associated p62

(A) 3.3 cells expressing an HA-tagged form of the *hump62* cDNA (Wong et al., 1992) were incubated (minus) for 30 hr in medium containing 0.5% fetal calf serum (FCS) and then stimulated (plus) with 20% fetal calf serum for 7 min. Immunoprecipitates obtained using anti-P (αP) antisera (defined in Figure 2A), plus or minus antigenic peptide, or with an antibody against the HA tag (BAbCO), were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with a pTyr antibody (Upstate Biotechnology Incorporated) (top) and, after stripping the filter, with anti–HA tag (αHA) (bottom). The positions of RasGAP, the RasGAP-associated proteins p62 and p190, the product of the epitope-tagged *hump62* cDNA (p62tag), and the immunoglobulin heavy chain (H chain) are indicated.

(B) Extracts of asynchronous (lanes a) or mitotically arrested (lanes m) 3.3 cells (treated with pervanadate before lysis) were prepared as described previously (Fumagalli et al., 1994). Proteins were isolated by immunoprecipitation with anti-C (αC) (Figure 2A), in the presence or absence of antigenic peptide, or with the anti–HA tag (αHA). Samples were immunoblotted with pTyr antibody (top) and, after stripping the filter, with the HA tag antibody (bottom). Arrows indicate the positions of the epitope-tagged *hump62* cDNA product (p62tag), Sam68, and the immunoglobulin heavy chain (H chain).

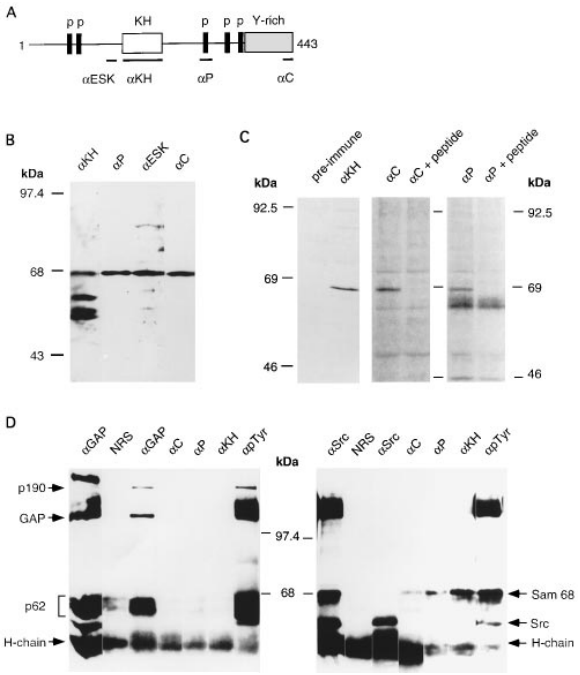

Figure 2. Sam68, but Not RasGAP-Associated p62, Is Recognized by Antisera against the Predicted Hump62 Product

(A) Schematic representation of the predicted Hump62 sequence (Wong et al., 1992) showing the five proline-rich motifs (p), the heterogeneous ribonucleoprotein K homology (KH) domain, and the Tyr-rich (Y-rich) C-terminus. The antisera anti-ESK, anti-KH, anti-P, and anti-C were raised in rabbits after immunization with a bacterial fusion protein or peptides corresponding to amino acids 136–148, 154–229, 291–311, and 437–443, respectively.

(B) Proteins were extracted from NIH 3T3 cells, separated by SDS–PAGE, transferred to nitrocellulose, and probed with the antisera indicated in (A).

(C) Total lysates of [^{35}S]methionine- and cysteine-labeled NIH 3T3 cells were immunoprecipitated with preimmune serum, anti-KH, anti-C, or anti-P antisera, with or without the corresponding antigenic peptides, and analyzed by SDS–PAGE and fluorography. (The protein[s] of approximately 62 kDa detected in the anti-P immunoprecipitate was probably due to nonspecific cross-reactivity since it was not blocked with the antigenic peptide.)

(D) Immunoprecipitates of GAP and associated proteins from an extract of asynchronous v-Src-transformed NIH 3T3 cells (first lane; left) or Src and associated proteins from an extract of mitotically arrested cells (first lane; right) were denatured by heating to 95°C. Individual components in the RasGAP (left) and Src immunocomplexes (right) were identified by a second round of immunoprecipitation with either normal rabbit serum (NRS), anti-C, anti-P, or anti-KH, or a pTyr antiserum (αpTyr). Proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and detected by immunoblotting with a pTyr antibody.

Tyr-phosphorylated p62 coimmunoprecipitated with RasGAP was reimmunoprecipitated with anti-pTyr antibodies, it was not detected with anti-C, anti-P, and anti-KH (Figure 2D, left), although the antibodies were effective in recovering Tyr-phosphorylated Sam68 coimmunoprecipitated with Src from mitotic extracts (Figure 2D, right). In summary, none of the antibodies raised against the predicted Hump62 protein detected RasGAP-associated p62 in any of the assays (although they reacted with Sam68). Since the antisera recognize widely separated epitopes within the Hump62 sequence, it seems likely that p62 and Sam68 are unrelated proteins.

The data presented explain the failure to detect p62 using antibodies generated to the deduced amino acid sequence of Hump62 (Ogawa et al., 1994; Taylor et al., 1995; Vogel and Fujita, 1995). At present, it is unclear how the purification of p62 with anti-pTyr antibodies lead instead to the isolation of Sam68. The molecular nature of RasGAP-associated p62 must await new purification and cloning strategies.

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